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Hepatocyte Wnts Are Dispensable During Diethylnitrosamine and Carbon Tetrachloride-Induced Injury and Hepatocellular Cancer

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Activation of the Wnt/β-catenin signaling is reported in large subsets of hepatocellular carcinoma (HCC). Upregulation of Wnt genes is one contributing mechanism. In the current study, we sought to address the role of hepatocyte-derived Wnts in a model of hepatic injury, fibrosis, and carcinogenesis. We subjected hepatocyte-specific Wntless knockout mice (HP-KO), unable to secrete Wnts from hepatocytes, and littermate controls (HP-CON) to diethylnitrosamine and carbon tetrachloride (DEN/CCl_4) and harvested at 3, 5, and 6 months for histological and molecular analysis. Analysis at 5 months displayed increased hepatic expression of several Wnts and upregulation of some, but not all, β-catenin targets, without mutations in Ctnnb1. At 5 months, HP-CON and HP-KO had comparable tumor burden and injury; however, HP-KO uniquely showed small $CK19^+$ foci within tumors. At 6 months, both groups were moribund with comparable tumor burden and CK19 positivity. While HCC histology was indistinguishable between the groups, HP-KO exhibited increased active β-catenin and decreased c-Myc, Brd4, E-cadherin, and others. Hepatic injury, inflammation, and fibrosis were also indistinguishable at 3 months between both groups. Thus, lack of Wnt secretion from hepatocytes did not affect overall injury, fibrosis, or HCC burden, although there were protein expression differences in the tumors occurring in the two groups.

Key words: Wnt; β-Catenin; Hepatocellular carcinoma; Diethylnitrosamine; Carbon tetrachloride

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common malignancy worldwide, with a median survival of 11 months and increasing incidence rates[1]. Although liver transplant is a promising treatment for a subset of patients, factors including lack of donor organs and failure to meet Milan criteria make transplant an unlikely option. FDA-approved therapies sorafenib and regorafenib, while helpful and limit HCC progression, extend patient survival by 3 months[2,3]. Further, HCC develops in cirrhotic livers in 70%–90% of cases, resulting from chronic liver injuries of all etiologies[4]. Because of increasing rates of liver diseases leading to fibrosis and tumorigenesis, the need for improved therapies to target and prevent HCC is growing.

β-Catenin signaling is upregulated in 20%–90% of HCC patients[5]. β-Catenin is part of the Wnt signaling pathway with many roles in liver pathophysiology[6]. β-Catenin activation in HCC can result from mutations in Ctnnb1, the gene encoding for β-catenin, or other mechanisms including overexpression of Wnt and its receptor Frizzled (Fzd)[7]. In fact, overexpression of many Wnts and Fzds has been implicated in different cancers including HCC[7–9] and several HCC cell lines[10]. These modifications result in increased nuclear accumulation of β-catenin, leading to transcriptional upregulation of target genes and promotion of tumorigenesis[9]. When Wnt antagonist fusion proteins are injected into an orthotopic HCC model harboring wild-type β-catenin, animal survival increases, and tumor volume, β-catenin activity, and angiogenesis

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decreases, suggesting that Wnts may be a promising therapeutic target[11]. There are Wnt inhibitors in phase I clinical trials for solid tumors, which may be effective for HCC patients with wild-type β-catenin (NCT01351103 and NCT01608867). Furthermore, patients with activated wild-type β-catenin resulting from Wnt or Fzd activation have more aggressive, dedifferentiated tumors and poorer prognosis than those with mutated β-catenin, yet less is known about the mechanism of wild-type β-catenin in tumor progression[8].

The main goal of our study was to assess hepatocytes as a source of Wnts, which may be essential in chronic liver injury, fibrosis, and HCC. We first confirmed a model of chronic injury [diethylnitrosamine and carbon tetrachloride (DEN/CCl_4)]-induced HCC and simultaneously induced transcriptional upregulation of Wnts, β-catenin activation, but not Ctnnb1 mutations, consistent with a previous report[12]. We next assessed whether Wnts from hepatocytes may be required for tumorigenesis after DEN/CCl_4. We utilized hepatocyte-specific Wntless knockout mice unable to secrete Wnts from hepatocytes (HP-KO)[13]. We subjected HP-KO and littermate controls (HP-CON) to DEN/CCl_4 until 5 months of age. Comparable tumors were observed in HP-KO and HP-CON, although we observed small $CK19^+$ foci in HP-KO. Overall, HCC behaved analogously in HP-KO and HP-CON up to 6 months despite increased activated β-catenin expression and decreased c-Myc, bromodomain 4 (Brd4), ERK1/2, Bax, and E-cadherin expression in HP-KO. We also assessed HP-CON and HP-KO at an early time point, but did not detect any differences in the injury microenvironment. Thus, our data suggest that hepatocyte-specific Wnts do not contribute to injury and fibrosis, but do participate in HCC development, and their loss leads to comparable tumor burden and histology in response to DEN/CCl_4.

MATERIALS AND METHODS

Animals

Animal work was performed in accordance with the Institutional Animal Care and Use Committee at the University of Pittsburgh. Albumin-Cre Wntless knockout mice were generated as described previously[13]. Male pups (HP-KO and HP-CON) were injected with 25 mg/kg diethylnitrosamine (Sigma-Aldrich) at 14–16 days prepared in sterile 0.9% saline. Animals were transferred to a BSL2+ facility and injected with 0.5 ml/kg carbon tetrachloride (CCl_4) twice per week from weeks 8 to 22, prepared in corn oil. Three days after the last injection, animals were sacrificed ($n=4$) and livers were harvested. Livers were also harvested from a precancer time point of 3 months ($n=3$) and advanced cancer time point of 6 months ($n=4$). Animals were monitored daily for signs of morbidity. At time of sacrifice, liver weights (LWs) and body weights (BWs) were assessed for LW/BW, and differences were tested for significance by Student's *t*-test with a value of $p<0.05$ considered significant. Blood was collected from the inferior vena cava, and serum was sent to the University of Pittsburgh Medical Center Clinical Laboratory for alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) testing.

Immunohistochemistry

Paraffin sections were processed as described elsewhere[14]. Sirius Red, Ki-67, hematoxylin and eosin (H&E), CD45, CK19, and glutamine synthetase (GS) staining was performed as also described in detail previously[14].

Protein Isolation and Analyses

Protein was extracted in RIPA buffer, quantified, and 30 μg was loaded onto a precast Bio-Rad SDS gel. Gels were transferred using Bio-Rad semidry transfer system. Antibody information can be found in Table 1. Horseradish peroxidase-conjugated secondary antibodies (Invitrogen) were used. Ponceau or GAPDH was used as a loading control. Densitometric analysis was performed with ImageJ and normalized to housekeeping control. Statistics were calculated using one-way ANOVA with multiple comparisons, and values of $p<0.05$ were considered significant.

For reverse-phase protein array (RPPA) analysis, samples were prepared via instructions from the MD Anderson Cancer Center RPPA Core Facility and sent for analysis. Data were analyzed via Student's *t*-test, and select proteins with statistically significant differences were validated by Western blot.

Table 1. List of Antibodies Used in the Study

Antibody	Company	Catalog Number
GS	Santa Cruz	Sc74430
c-Myc	Santa Cruz	Sc764
Hypophosphorylated β-catenin	Cell Signaling	4270
PCNA	Santa Cruz	Sc56
Cyp2e1	Atlas Antibodies	HPA009128
β-Catenin	BD Biosciences	610154
GAPDH	Proteintech	60004-1-Ig
Wee1	Cell Signaling	4936
Yap	Cell Signaling	4912
ERK1/2	Cell Signaling	4695
Bax	Cell Signaling	2772
Cdc25	Cell Signaling	4688
Brd4	Cell Signaling	13440
E-cadherin	Cell Signaling	3195
PKAa	Cell Signaling	5675

RNA Isolation and qPCR

RNA was extracted from DEN/CCl_4-treated control livers and untreated livers ($n=3$) using TRIzol (Thermo Fisher), and after DNAse treatment (Thermo Fisher), 2 μg of RNA was reverse transcribed using SuperScript III (Thermo Fisher). Samples were pooled together, and SYBR Green (Thermo Fisher) was used for qPCR. Ct values were normalized to GAPDH, and primer sequences are included in Table 2.

DNA Extraction and Ctnnb1 Sequencing

gDNA was isolated from livers (XNAT; Sigma-Aldrich), and primers flanking intron–exon junctions of Ctnnb1 exon 2 described previously[15] were used in PCR. Product was gel purified and sequenced by the Genomics Research Core at University of Pittsburgh. Sequences were validated using ApE Software. Tumors in HP-CON and HP-KO at 5 and 6 months ($n=3$) were sequenced.

RESULTS

DEN/CCl_4 Leads to Wild-Type Ctnnb1 and Upregulation of Several Wnts

To develop an injury-based carcinogenesis model, we adapted the DEN/CCl_4 protocol used to induce inflammation, fibrosis, and tumors[16,17]. This model utilizes one injection of diethylnitrosamine at day 15, and biweekly injections of carbon tetrachloride from week 8 to week 22 (DEN/CCl_4) (Fig. 1A). DEN/CCl_4 shows 100% penetrance at 5 months for HCC in conjunction with fibrosis and inflammation, thereby representing what is seen in patients (Figs. 1B and 2E).

This model is known to cause HCC without β-catenin mutations as confirmed through sequencing and supported by lack of GS staining[12], which is a surrogate, albeit debated, for mutated β-catenin[18,19]. Via immunohistochemistry, all tumors were GS^- (Fig. 1C). Further, tumors were sequenced for mutations in exon 2 of Ctnnb1, analogous to exon 3 in humans. This is home to GSK3β phosphorylation sites and the most frequent activating mutations in HCC[15,20]. Wild-type Ctnnb1 was identified in all tumors (Fig. 1D). This was also corroborated using microarray data obtained from Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), accession number GSE33446, comparing data from DEN/CCl_4-treated animals to untreated animals[16]. We queried the data and noted no change in expression of Glul, gene encoding for GS, after DEN/CCl_4. However, several β-catenin targets were upregulated as listed in Table 3, suggesting that modest activation of the pathway does occur despite absent Ctnnb1 mutations.

Next, we assessed whether Wnts were overexpressed after DEN/CCl_4 and tested mRNA expression of select Wnts in livers after DEN/CCl_4 compared to untreated livers. Wnt1, Wnt6, Wnt10a, Wnt10b, Wnt11, and Wnt16 showed upregulation of at least twofold in DEN/CCl_4 livers versus control livers (Fig. 1E). We hypothesized that at least some of these may be originating from hepatocytes and hence interrogated tumorigenesis in mice lacking ability to secrete all Wnts from hepatocytes owing to Wntless loss.

HP-KO and HP-CON Have Comparable Tumor Burden at 5 Months, Although HP-KO Has More $CK19^+$ Nodules After DEN/CCl_4

To assess the role of Wnts from hepatocytes, we subjected previously described hepatocyte-specific Wntless KO (referred henceforth as HP-KO) and littermate controls (HP-CON) to DEN/CCl_4[13]. Mice sacrificed at 5 months exhibited comparable tumor burden reflected by similar LW/BW between the two groups (Fig. 2A and B). Serum analysis revealed comparable and modestly elevated ALT, AST, and ALP in HP-CON and

Table 2. List of qPCR Primers Used in the Study

Primer (Mouse)	Forward Sequence	Reverse Sequence
GAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA
Wnt1	ATCCATCTCTCCCACCTCCTAC	GAATCTTTCTCTCACCCTCTGG
Wnt2	TCTGTCTATCTTGGGCATTCTG	TTCCTTCGCTATGTGATGTTTC
Wnt2b	ACCTTCCTCTACCCTCAATCCT	TCACTCAGCCTCCTAAATCCAT
Wnt3	GTCTGCTAATGCTGGCTTGAC	TAGGAAGGGATGGGAGGTGT
Wnt4	AGAACTGGAGAAGTGTGGCTGT	AAAGGACTGTGAGAAGGCTACG
Wnt6	TTTACACCAGCCCACGAAAG	ACTCACCCATCCATCCAAGTA
Wnt8b	GTTTGCTTGGGACCGTTG	TCCATTTCGGGAGTCATCA
Wnt9a	ATGGTGTGTCTGGCTCCTG	CAGTGGCTTCATTGGTAGTGCT
Wnt9b	GGGTGTGTGTGGTGACAATCT	GGTCCTTGCTTCCTCTCTTG
Wnt10a	TCCTGTTCTTCCTACTGCTGCT	ACGCACACACACCTCCATC
Wnt10b	CCACTACAGCCCAGAACCTC	GGAGAGACCCTTTCAACAACTG
Wnt11	CCCTGGAAACGAAGTGTAAATG	AGGTAGCGGGTCTTGAGGTC
Wnt16	GCTGTAACCTCCTCTGCTGTG	GTGGACATCGGTCATACTTTCA

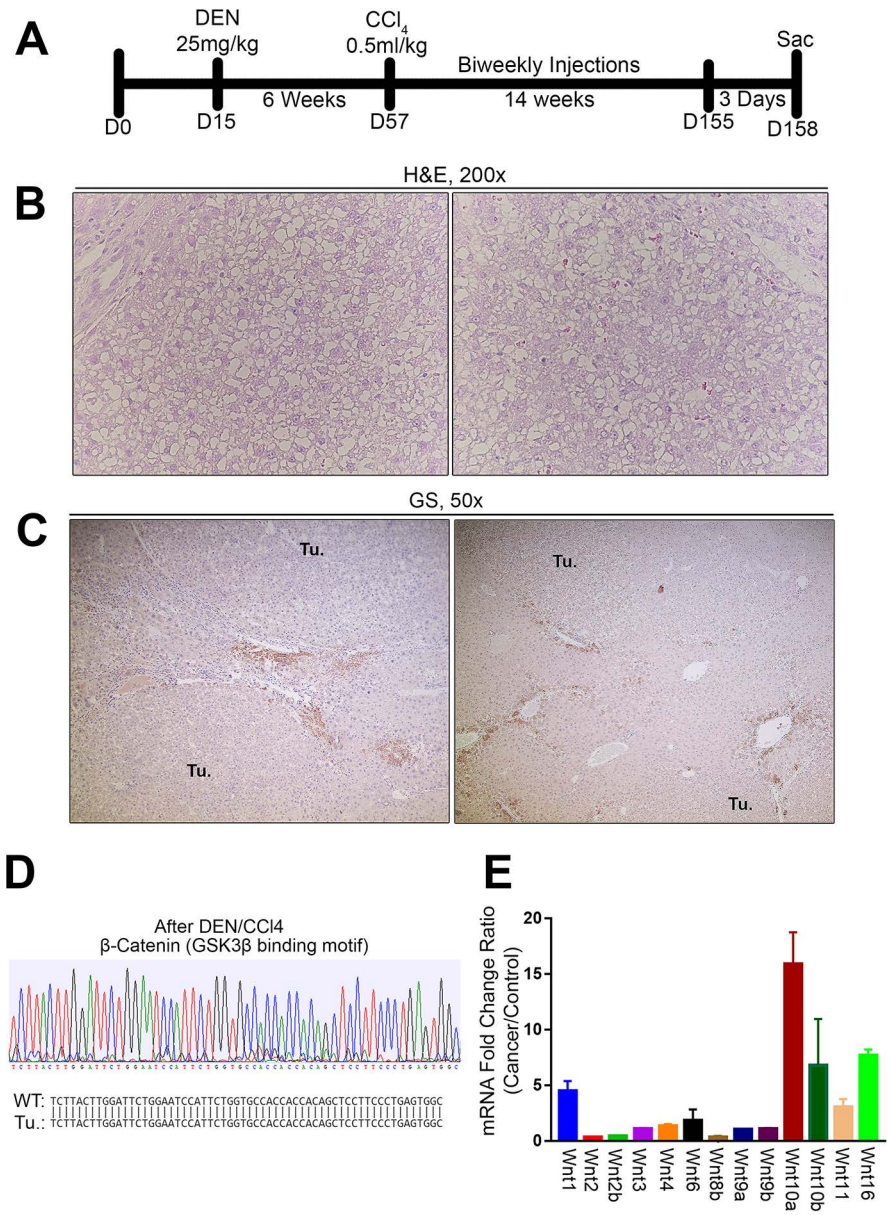

Figure 1. The diethylnitrosamine and carbon tetrachloride (DEN/CCl_4) model of hepatocellular carcinoma (HCC) shows Wnt upregulation but nonmutated β-catenin (A) Schematic of DEN/CCl_4 injection protocol, beginning at day of birth and concluding at 5 months of age. (B) High magnification (200×) hematoxylin and eosin stain (H&E) highlighting intratumoral dysplastic hepatocytes. (C) Assessing glutamine synthetase (GS) staining in tumors in the DEN/CCl_4 model of HCC. No GS^+ tumors were observed, suggesting wild-type β-catenin. (D) Representative sequencing of β-catenin exon 2, focusing on GSK3β phosphorylation motifs, displays nonmutated and identical sequence between tumors (Tu.) and wild type (WT). (E) RT-PCR analysis showing upregulation in the transcript levels of several Wnts at 5 months of age after continued DEN/CCl_4 treatment in mice as compared to untreated controls.

HP-KO (Fig. 2C–E). H&E staining verified these tumors to be similar HCCs as reflected by well-circumscribed lesions composed of hepatocytes with basophilic cytoplasm, some pleomorphic nuclei, and mitotic figures in HP-CON and HP-KO (Fig. 2F). Further, we noted no differences in fibrosis via Sirius red or α-SMA (Fig. 2F and data not shown), or inflammation, vascularization, or proliferation, via assessment of CD45, CD31, and Ki-67, respectively (Fig. 2F and data not shown). We also validated wild-type Ctnnb1 in HP-CON and HP-KO (Fig. 2G).

To further characterize the tumor phenotype, we queried the differentiation status and assessed the dedifferentiation marker CK19, as it positively correlates

Table 3. List of β-Catenin Target Genes Altered in the DEN/CCl_4 Model of Injury, Fibrosis, and HCC

Gene	Probe (from GSE33446)	Log2 Fold Change	Adjusted *p* Value	Reference*
CD44	1434376_at	3.55382771	8.71e-03	36
Survivin	1424278_a_at	3.36261188	6.73e-04	37
EGFR	1432647_at	2.58970608	3.94e-05	38
Ccnd1 (cyclin D1)	1417419_at	2.46649404	4.01e-09	39
S100A6	1421375_a_at	2.40064056	8.99e-03	40
Ctnnb1 (β-catenin)	1430533_a_at	2.112623	5.53E-06	
MMP14	1448383_at	2.06551913	4.32e-06	41
Vegfa	1451959_a_at	0.7674417	2.87e-03	42
Tgfα	1450421_at	0.50142774	6.94e-04	43
Axin2	1436845_at	0.39369432	7.48e-01	44
Lef1	1421299_a_at	0.11541357	4.32e-01	45
Glul (GS)	1426236_a_at	−1.21199767	2.21e-01	46
Regucalcin	1448852_at	−1.28147025	7.64e-02	47
Lect2	1449492_a_at	−1.35084301	3.03e-01	48

The data were obtained from microarray data available in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/), accession number GSE33446, and values presented are ratio of the expression of genes in DEN/CCl_4-treated versus untreated mice[16].
*References are to the studies showing the gene as being target of the Wnt/β-catenin signaling pathway.

with poor prognosis, increased tumor size, and invasiveness[21]. Immunohistochemistry revealed small $CK19^+$ foci in all HP-KO mice, while expression was restricted to bile ducts in HP-CON mice (Fig. 3A). Not all nodules in HP-KO were $CK19^+$; however, large tumors frequently contained smaller foci of heterogeneous $CK19^+$ cells. These overall findings indicated that HCC occurring in the absence of Wnts from hepatocytes were comparable to controls, other than the occurrence of small $CK19^+$ areas within tumors.

No Difference in Tumor Burden in HP-KO Versus HP-CON at Advanced Stages

We predicted $CK19^+$ foci in HP-KO to develop into more aggressive tumors than HP-CON. To test this, we followed an independent cohort for an additional time frame. However, by 6 months, all mice became moribund and required euthanasia. Grossly, livers from both groups were excessively stiff and showed sizable tumor burden (Fig. 3B). LW/BW at 6 months failed to show significant differences between the two groups, and both groups showed increased ratios over the 5-month time point (Fig. 3C). Mutations in Ctnnb1 were still absent at 6 months (Fig. 3D). Intriguingly, at 6 months, both HP-CON and HP-KO displayed comparable, diffuse, and broader staining for CK19 within tumors (Fig. 3E). Similar to the 5-month time point, comparable fibrosis, inflammation, and proliferation were observed as seen by Sirius Red (Fig. 3E), CD45, and Ki-67 staining, respectively (data not shown). Thus, there were no phenotypic differences in HCC occurring in HP-CON and HP-KO at 6 months after DEN/CCl_4.

HP-CON and HP-KO After DEN/CCl_4 Show Distinct Temporal Protein Expression

Despite comparable tumor burden and histology in HP-CON and HP-KO at 5 and 6 months, we queried whether there are differences in protein expression, likely correlating with altered signaling patterns, as a result of absent Wnt secretion from hepatocytes. First, we assessed proliferation markers and relevant β-catenin activation markers via Western blot (Fig. 4A and C). Hypophosphorylated β-catenin, suggesting activation, was comparable at 5 months and significantly upregulated in HP-KO at 6 months. GS was variable, while Cyp2e1, a β-catenin target altered by CCl_4, was lower in HP-KO at 5 months, although levels in both HP-CON and HP-KO were comparably reduced at 6 months. Total levels of β-catenin were comparably reduced at 6 months compared to 5 months. Cyclin D1 was increased at 6 months compared to 5 months in both groups. PCNA remained comparably high at all times in both groups. Intriguingly, c-Myc was reduced in HP-KO at both 5 and 6 months. Thus, overall hepatocyte-specific loss of Wnts appears to promote β-catenin hypophosphorylation and decrease c-Myc levels after DEN/CCl_4.

We sought a holistic approach to assess protein levels and performed RPPA to assess expression of over 240 proteins involved in cancer as described in Materials and Methods. Several proteins had significant differences between HP-CON and HP-KO in the 5-month or 6-month time point, and select proteins were confirmed by Western blot (Fig. 4B and D). Intriguingly, at 5 months, HP-KO had significantly less expression of ERK1/2, Bax, Brd4, and E-cadherin than HP-CON. By 6 months, HP-KO

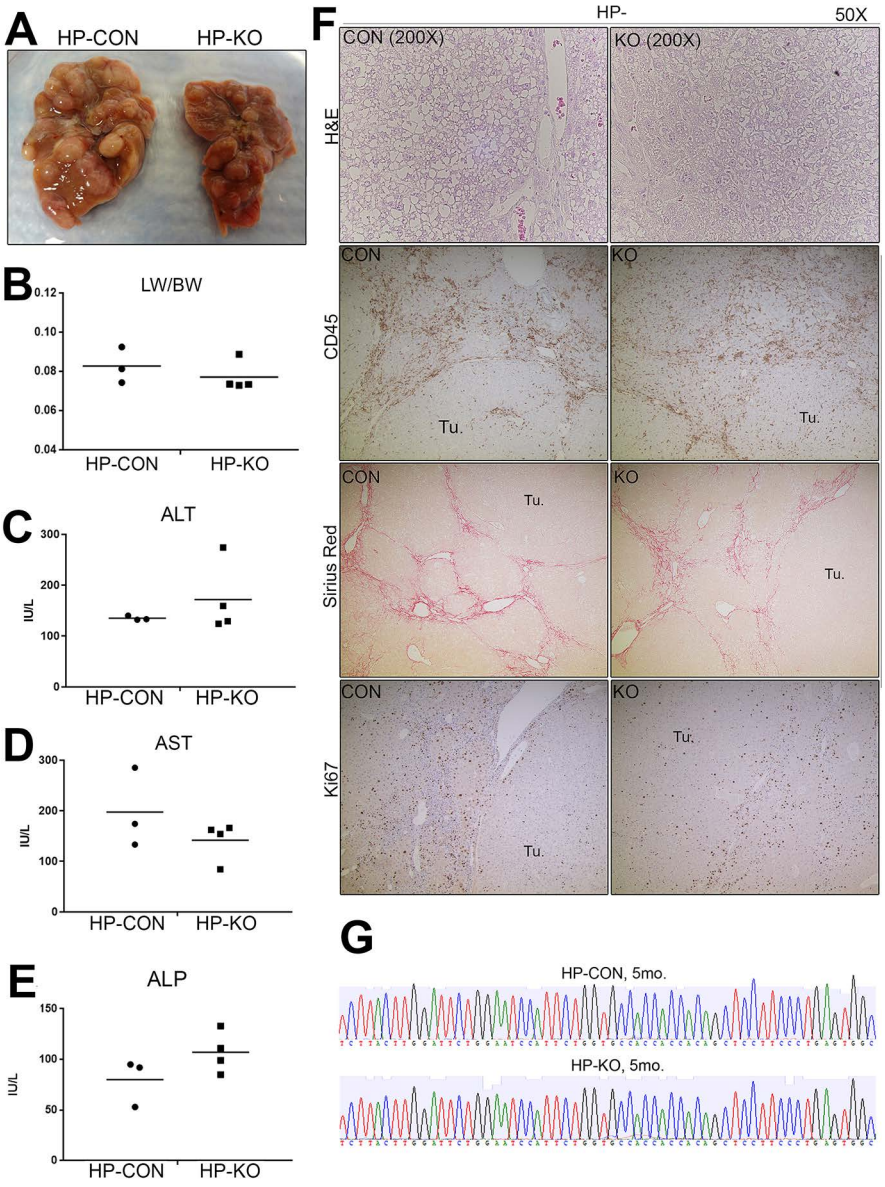

Figure 2. Littermate controls (HP-CON) and hepatocyte-specific Wntless knockout mice (HP-KO) have comparable tumor burden after 5 months of DEN/CCl_4. (A) Representative gross images of HP-CON and HP-KO at 5 months. (B) HP-CON and HP-KO have comparable liver weight-to-body weight ratios after DEN/CCl_4. (C–E) Serum analysis reveals comparable alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) in HP-CON and HP-KO. (F) Representative images of H&E (200×), CD45, Sirius Red, and Ki-67 (50×) reveal comparable tumors and injury burden in HP-CON and HP-KO. (G) Representative sequencing shown for HP-CON and HP-KO tumors, which are both CTNNB1 wild type.

had increased ERK1/2 and decreased Brd4, and comparable Bax and E-cadherin. Expression of Wee1, Yap, and Cdc25 was variable, and while PKAa expression was only evident at 6 months, there was no difference between HP-CON and HP-KO (Fig. 4B and D). Taken together, this suggests HP-specific Wnts temporally regulate expression of oncogenes including ERK1/2, Bax, E-cadherin, and Brd4.

Comparable Onset and Progression of Injury in Response to DEN-CCl_4 in HP-KO and HP-CON

Last, we asked whether hepatocyte Wnts contribute to DEN/CCl_4-induced injury and hence the pretumoral microenvironment. We subjected HP-CON and HP-KO to this insult until 3 months of age and noted no gross hepatic differences (Supplemental Fig. 1A, available at

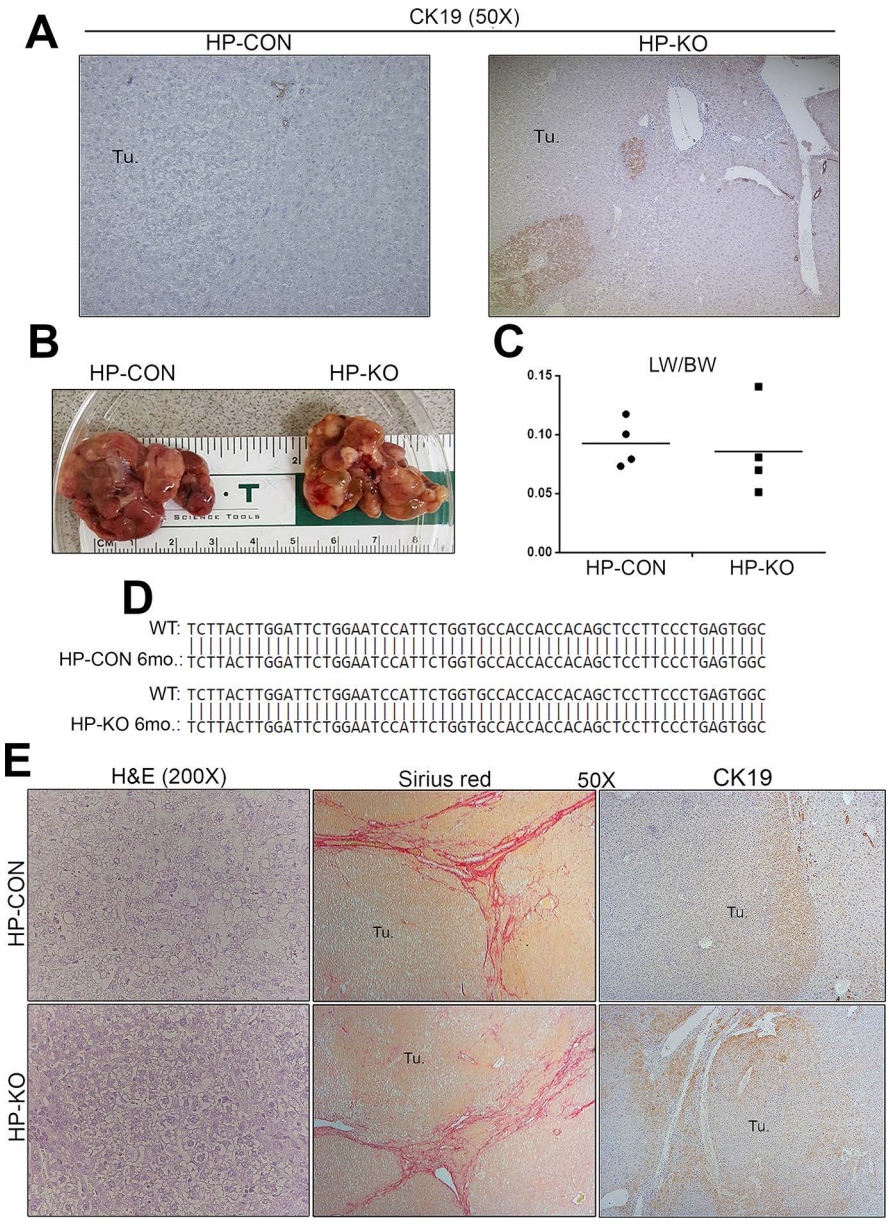

Figure 3. HP-KO have CK19$^+$ foci absent in HP-CON at 5 months; however, HCC is comparable in HP-CON and HP-KO at 6 months after DEN/CCl_4. (A) CK19 staining is positive in biliary epithelial cells in HP-CON and HP-KO, but is evident in small tumor foci within larger tumors in HP-KO at 5 months. (B) Gross images show advanced HCC in both HP-CON and HP-KO at 6 months after DEN/CCl_4. (C) Liver weight-to-body weight ratios are comparable at 6 months after DEN/CCl_4. (D) At 6 months, HP-CON and HP-KO have wild-type CTNNB1. (E) Representative H&E (200×), Sirius Red (50×), and CK19 (50×) suggest similar tumor composition, fibrosis, and CK19 pattern, respectively, in HP-CON and HP-KO at 6 months.

https://drive.google.com/open?id=1zbEhnTG20BouorVTHpxAb8RdmmJoTBTS). Histology showed comparable cell death, hepatocyte ballooning, inflammation, and scarring in HP-CON and HP-KO (Supplemental Fig. 1B). Further analysis revealed similar fibrosis, inflammation, and proliferation via Sirius Red, number of CD45$^+$ cells, and number of Ki-67$^+$ hepatocytes in HP-CON and HP-KO (Supplemental Fig. 1C). We therefore concluded that removing Wntless from HP insignificantly contributes to hepatic injury during DEN/CCl_4 treatments.

DISCUSSION

Activation of Wnt/β-catenin signaling in HCC is due to various mechanisms[5,22]. Overexpression of Wnt

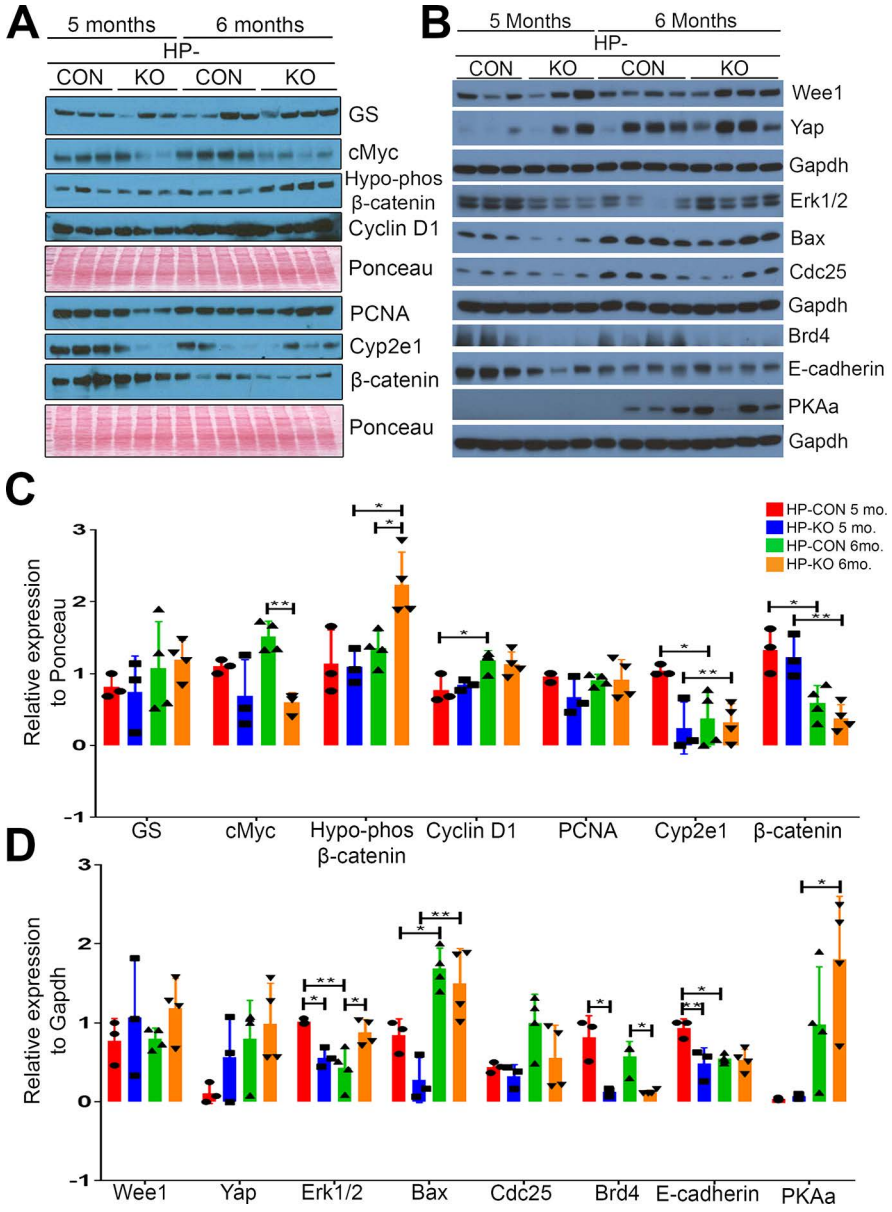

Figure 4. HP-CON and HP-KO have divergent protein expression patterns at 5 and 6 months. (A) Western blot assessing GS, c-Myc, hypophosphorylated β-catenin, cyclin D1, PCNA, Cyp2e1, and total β-catenin. Ponceau shows comparable loading. (B) Western blot assessing select proteins based off reverse-phase protein array (RPPA) analysis including Wee1, Yap, ERK1/2, Bax, Cdc25, bromodomain 4 (Brd4), E-cadherin, and PKAa. GAPDH shows comparable loading. (C) Densitometry of Western blot from (A) highlights significant changes between HP-CON and HP-KO at 5 and 6 months. Relative expression was normalized to Ponceau. (D) Densitometry analysis of Western blot from (B), normalizing values to GAPDH. Statistics performed with one-way ANOVA using multiple comparisons. *$p<0.05$, **$p<0.01$.

and Fzd genes has been implicated as a contributor to β-catenin activation in a subset of HCC cases[7]. In fact, patients with intratumoral overexpression of Wnt and/or Fzd have more dedifferentiated tumors correlating with aggressiveness[8]. Wnt upregulation has also been reported in HCC cell lines including Hep3B cells[10]. To address the role of hepatocyte-derived Wnts in hepatocarcinogenesis in a relevant model, we examined injury and tumorigenesis in control mice and mice lacking Wntless in hepatocytes, which disallows secretion of all Wnts from these cells due to loss of this cargo receptor specific and essential for Wnts[13,23,24].

We studied the DEN/CCl_4 model of tumorigenesis, as it leads to HCC following chronic injury and fibrosis,

mimicking patient progression[17]. More importantly, we confirmed that DEN/CCl_4-induced HCC provides evidence of β-catenin activation without mutations in Ctnnb1. Indeed, microarray data available publicly via Gene Expression Omnibus (GSE33446) confirm that β-catenin target genes including CD44, cyclin D1, epidermal growth factor receptor (EGFR), and survivin are induced in this model, whereas β-catenin targets like GS and Regucalcin, which are associated with more sustained β-catenin activation through mutations, are unchanged. Further, when mRNA expression of several Wnts was assessed, Wnt1, Wnt6, Wnt10a, Wnt10b, Wnt11, and Wnt16 were upregulated at least twofold in comparison to control livers. While in the current study we addressed the loss of all hepatocyte-derived Wnts, studying the relevance of individual Wnts will be interesting, especially since Wnt6 and Wnt11 can act noncanoncially, independent of β-catenin[25].

Upon challenging HP-CON and HP-KO with DEN/CCl_4, comparable tumor burden was observed at 5 months. We noted similar injury, fibrosis, inflammation, and proliferation. However, in assessing tumor differentiation, we observed the presence of small but frequent $CK19^+$ foci within larger $CK19^-$ tumors in HP-KO. Presence of CK19 in HCC is suggested to correlate with a more dedifferentiated phenotype, overall poor prognosis, and worse outcome after surgery[26–28]. Finding $CK19^+$ foci in HP-KO was surprising, since a positive correlation between Wnt/β-catenin activation and stem markers including CK19 has been previously reported[29]. However, since secretion of all Wnts is being disrupted, it is likely that the loss of noncanonical Wnts may be allowing for increased CK19 positivity. Indeed, hepatocytes and transformed hepatocytes are known to be a source of noncanonical Wnts that suppress β-catenin activity[10]. Intriguingly, the increased numbers of $CK19^+$ foci did not lead to an overall aggressive HCC in HP-KO at 6 months, and both groups of mice succumbed to excessive tumor burden, and CK19 staining was comparable at this time point. These data suggest that the DEN/CCl_4 model is too robust, leading to an aggressive disease, and a model with an indolent course may be more suitable to address the overall role of hepatocyte-derived Wnts.

We also noted a reduction in c-Myc in HP-KO at 5 months and 6 months. Wnt/β-catenin activates c-Myc, particularly during hepatocarcinogenesis[30]. Therefore, a reduction in c-Myc would likely suggest a reduction in β-catenin activity. While β-catenin target gene Cyp2e1 was reduced in HP-KO at 5 months, target gene GS and hypophosphorylated and total β-catenin levels were unchanged. Although at 6 months a reduced c-Myc in HP-KO correlated with increased hypophosphorylated (and thus activated) β-catenin. c-Myc can be upregulated in HCC independent of β-catenin, for example, by amplification[31]. The mechanism by which c-Myc is downregulated in HP-KO remains unclear and will be elucidated in the future. Overall, decreased c-Myc does not seem to be altering the overall tumor biology in HP-KO since comparable tumor burden is evident in HP-KO and HP-CON. However, c-Myc upregulation in HP-CON does appear to be partially Wnt/β-catenin dependent as shown previously[30,32].

RPPA analysis further revealed altered protein expression in HP-KO versus HP-CON. While the relevance of these changes remains unclear, there are several intriguing relationships among these data. Brd4 is a transcription coactivator involved in many cancers. In HCC, Brd4 is overactive, and its suppression correlates with c-Myc suppression[33]. At the 5-month and 6-month time points, c-Myc and Brd4 were both reduced in HP-KO. Whether HP-Wnts regulate c-Myc through Brd4 remains a plausible mechanism to assess. E-cadherin inversely correlates with aggressive HCC, as loss leads to increased epithelial-to-mesenchymal transition and invasiveness[34]. Reduction of E-cadherin in HP-KO at 5 months, then comparable expression to HP-CON at 6 months, supports the findings that at 5 months HP-KO tumors began to dedifferentiate, but all differences are ablated by 6 months. This likely reaffirms that the model is too hepatotoxic to appreciate contributions of HP Wnts, as other pathways are able to compensate. E-cadherin can activate ERK signaling in other cancers[35], which may explain why reduced E-cadherin correlates with reduced ERK1/2 at 5 months; however, further studies are required to confirm pathway activation. Finally, the role of HP-Wnts in Bax expression is unclear, despite HP-KO having reduced Bax at 5 months. Overall, we conclude that HP-Wnts contribute to protein expression, which likely corresponds to signaling changes but are insignificant to the overall tumor phenotype.

Wnt signaling from hepatocytes also appears to not influence the overall pretumor environment. At 3 months, comparable injury, inflammation, and fibrosis were observed between HP-CON and HP-KO. These negative data are novel and important, as we provide in vivo evidence that targeting hepatocyte-specific Wnts will not be an effective clinical therapy, despite literature demonstrating increased Wnts in HCC and transformed hepatocytes in vitro. It will be of interest to assess Wnt contributions from additional cell types, including macrophages and endothelial cells, to identify roles to injury, fibrosis, and carcinogenesis.

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